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14. ABSTRACT
Abnormalities in programmed cell death (apoptosis) machinery play a crucial role in initiation, progression and metastasis of prostate cancer. Therefore, molecules that initiate pro-apoptotic pathways are excellent therapeutic agents in prostate cancer. However, some prostate cancer cells develop resistance to pro-apoptotic agents. In this proposal we are examining the regulatory mechanisms of c-FLIP(L), which is an important modulator of apoptosis in prostate cancer.

15. SUBJECT TERMS
Prostate cancer, TRAIL-induced apoptosis, HGS-ETR2, c-Fos, gene regulation of c-FLIP(L)

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Progress Report
November 2006 to November 2007

INTRODUCTION

Abnormalities in apoptotic machinery play a crucial role in initiation, progression and metastasis of prostate cancer. c-FLIP(L), an anti-apoptotic molecule, has been suggested to play a role in developing resistance to pro-apoptotic agents like tumor necrosis factor (TNF) and TNF related apoptosis inducing ligand (TRAIL). In this proposal we have demonstrated that expression of c-FLIP(L) is necessary and sufficient to account for developing resistance to pro-apoptotic agents like TRAIL. Silencing expression of c-FLIP(L) is adequate to overcome other alternative mechanisms of resistance to TRAIL. Therefore, changing the expression of c-FLIP(L) successfully converts the phenotype of resistant to sensitive prostate cancers in response to pro-apoptotic agents. In addition, the expression of c-FLIP(L) is partially regulated at the transcriptional level.

Two pro-agonist antibodies to the TRAIL receptors have been developed by the Human Genome Science, Inc. (Rockville, MD) designated HGS-ETR1 and HGS-ETR2. HGS-ETR and HGS-ETR2 target the DR4 and DR5 TRAIL receptors. Both of these newly developed drugs are presently in phase I and phase II clinical trials in other carcinomas. We have obtained approval from Human Genome Science's scientific committee review board to use HGS-ETR1 and HGS-ETR2 in our prostate cancer studies outlined below. Therefore, knowledge gained from this proposal directly translates to identifying prostate cancer patients who may benefit the most from the pro-apoptotic effects of HGS-ETR1 and HGS-ETR2. This proposal is focused on identifying the molecular mechanisms of resistance to the pro-apoptotic effects of HGS-ETR1 and HGS-ETR2.

This is a progress report for November 2006 to November 2007. However, please note that this grant was recently transferred from Beth Israel Deaconess Med Center to Massachusetts General Hospital and the funds transferred only became available recently, therefore, I have requested and obtained permission for a one year no-cost extension for this grant from November 2007 to November 2008.

Recently, an interim report was submitted to the Department of Defense, and this report, will contain much of the same content, since this transfer of funds only became available within the last month.

BODY

Specific Aim #1: To examine the efficacy of HGS-ETR1 and HGS-ETR2 in an orthotopic prostate cancer model.

Previously we developed a prostate cancer orthotopic model, and demonstrated efficacy of HGS-ETR2 for TRAIL-sensitive cells (please see last Progress Report). Since our last progress report, we have been correlating our in-vivo findings with some in-vitro experiments. We have utilized cell death assays (Annexin V and MTT assays) to demonstrate that PC3 cells are sensitive to HGS-ETR2 therapy. In contrast, PC3 cells are not very sensitive to HGS-ETR1 treatment. Subsequently, we have demonstrated that HGS-ETR2 represses the expression of the anti-apoptotic molecule, c-FLIP(L), a molecular mechanism that is similar to our previous findings using recombinant TRAIL (1).

Previously we have shown that c-Fos/AP-1 promotes TRAIL(or HGS-ETR2)-induced apoptosis by repressing the anti-apoptotic molecule c-FLIP(L). We have found that activation of c-Fos is necessary, but insufficient for apoptosis. In this portion of our studies we investigated whether synthetic induction of c-Fos/AP-1 by low-dose 12-O-Tetradecanoylphorbol-13-acetate (TPA) (2, 3) converts the phenotype of TRAIL-resistant prostate cancer cells to a TRAIL-sensitive phenotype. Recently, we found that HGS-ETR2 when combined with TPA effectively reduces the tumor volume of resistant prostate cancer xenografts. We have examined molecular changes of the xenografts that have been treated with HGS-ETR2 and TPA. We have found that in the presence of TPA, c-Fos is upregulated, while c-FLIP(L) is down-regulated in order to sensitize resistant LNCaP xenografts to the pro-apoptotic effects of HGS-ETR2. Recently, we have demonstrated that c-Fos functions as a pro-apoptotic agent by repressing c-FLIP(L) (4-6). In our in-vivo xenograft studies, we have found that treatment with TPA enhances expression of c-Fos, represses the expression of c-FLIP(L) and sensitizes HGS-ETR2 resistant xenografts (Figs. 1 & 2).

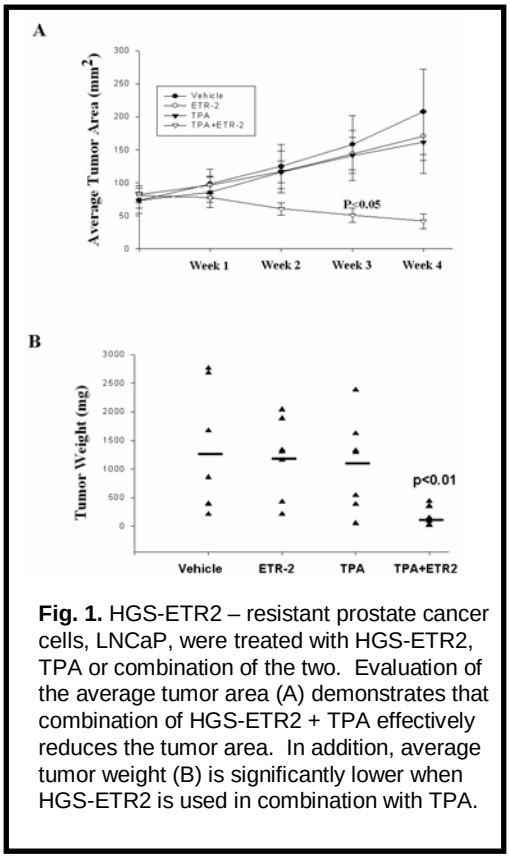


Fig. 1. HGS-ETR2 – resistant prostate cancer cells, LNCaP, were treated with HGS-ETR2, TPA or combination of the two. Evaluation of the average tumor area (A) demonstrates that combination of HGS-ETR2 + TPA effectively reduces the tumor area. In addition, average tumor weight (B) is significantly lower when HGS-ETR2 is used in combination with TPA.

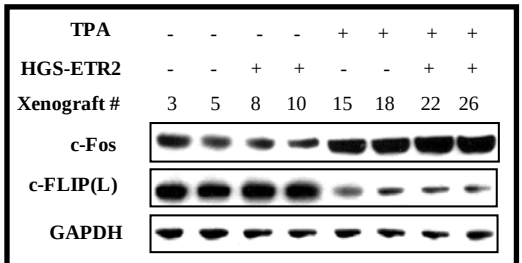
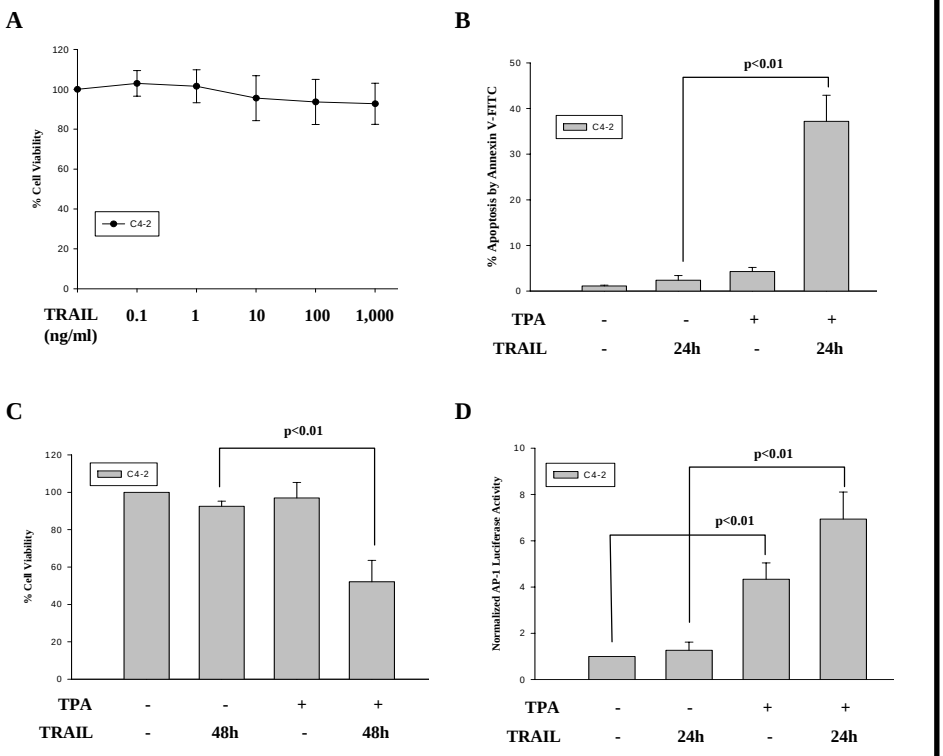


Fig. 2. TPA up-regulates c-Fos and represses c-FLIP(L) protein levels in LNCaP xenografts.

TPA enhances TRAIL-induced apoptosis in androgen-independent prostate cancer cells

Since majority of the morbidity and mortality associated with prostate cancer is secondary to progression from an androgen dependent to an androgen independent state and inefficacy of currently available systemic regimens, we wished to examine whether combination of TPA and TRAIL is effective against TRAIL-resistant androgen independent prostate cancer cells. High dose TPA has been shown to induce apoptosis

Figure 3

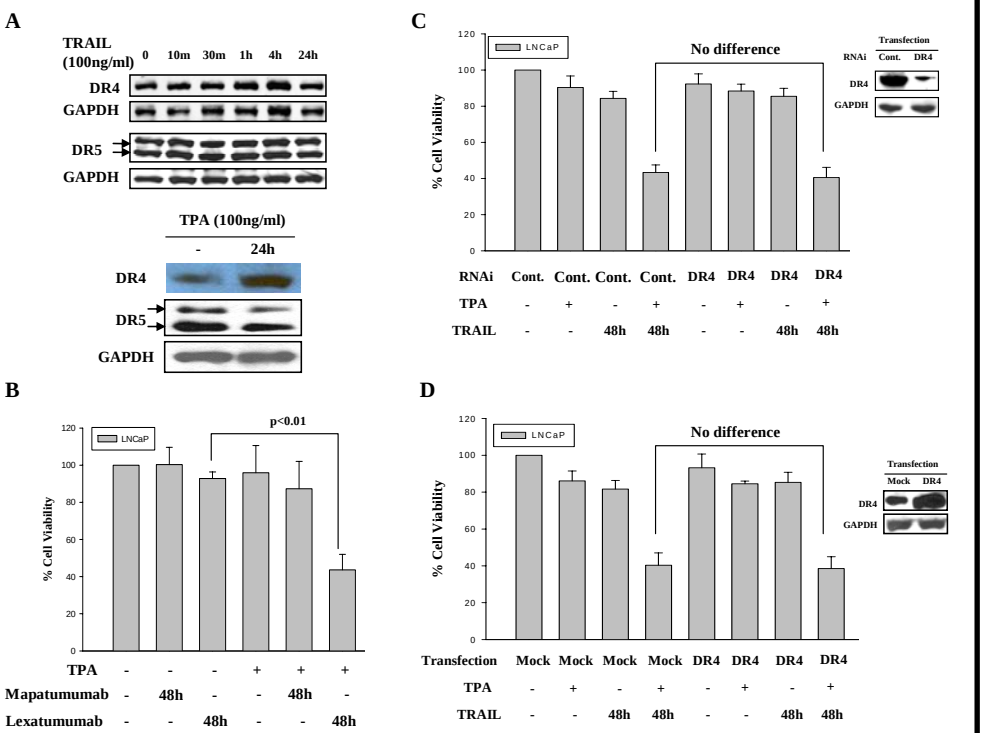


in the androgen-dependent LNCaP prostate cancer cells, while, TPA is thought to be ineffective in androgen-independent prostate cancer cells (7, 8). Since high dose TPA is associated with tumor progression properties (9), we wished to determine whether low-dose TPA when combined with TRAIL can also sensitize androgen independent prostate cancer cells that are resistant to TRAIL-induced apoptosis. We found that C4-2 cells, an androgen independent subline of LNCaP cells (10), were resistant to TRAIL-induced apoptosis – a finding similar to the parental LNCaP cells which are androgen dependent (Fig. 3A). Next, we wished to determine whether TPA can enhance the pro-apoptotic properties of TRAIL. We found that combination of TPA with TRAIL converted the phenotype of TRAIL-resistant C4-2 cells to a TRAIL-sensitive phenotype as evidenced by increased apoptotic rate (Fig. 3B) and reduced cell viability (Fig. 3C). In concert with our previous findings in the androgen dependent LNCaP cells, enhancement of TRAIL-induced apoptosis was associated with increased AP-1 activity in the androgen independent C4-2 cells (Fig. 3D). Therefore, low dose TPA is capable of converting the phenotype of TRAIL-resistant prostate cancer cells in both androgen dependent and independent states by increasing AP-1 gene activity.

TPA enhancement of TRAIL-induced apoptosis is independent of DR4 levels

Treatment of LNCaP cells with TRAIL does not alter the protein levels of the DR4 or DR5 TRAIL receptors (Fig. 4A, upper panel). Some investigators have suggested that TPA enhances the expression of TRAIL receptor DR4 via an AP-1 dependent mechanism (11). However, it is unclear whether increased levels of DR4 are associated with enhancement of TRAIL-induced apoptosis. We, similar to others (11), found that TPA increased TRAIL receptor DR4 levels, but not DR5 levels (Fig. 4A, lower panel).

Figure 4



Next we wished to determine whether DR4 and/or DR5 play a functional role in TPA enhanced TRAIL-induced apoptosis. Since recombinant TRAIL activates both DR4 (TRAIL-R1) and DR5 (TRAIL-R2) by promoting trimerization of these cell surface receptors, we utilized fully human monoclonal agonist antibodies specifically targeted against TRAIL-R1 (Mapatumumab) and TRAIL-R2 (Lexatumumab) (12, 13). We found that Mapatumumab alone or in combination with TPA did not enhance TRAIL-induced cell death in LNCaP cells (Fig. 4B). In contrast, Lexatumumab, when combined with TPA promoted cell death and reduced cell viability in the TRAIL-resistant prostate cancer cells (Fig. 4B), which is similar to the results using soluble TRAIL combined with TPA and compatible with our xenograft in-vivo studies (Fig. 1). Further, we examined whether inhibition of DR4 by RNAi (Fig. 4C – inset) or ectopic expression of DR4 (Fig. 4D - inset) would alter sensitivity to TRAIL-induced apoptosis. We found that neither inhibition of DR4 (Fig. 4C), nor increased DR4 levels (Fig. 4D), were associated with TRAIL-induced or TPA-enhanced TRAIL-induced cell death (Fig. 4C and 4D). Conversely, overexpression of DR5 alone sensitized LNCaP cells to TRAIL-induced apoptosis, which was even further enhanced when TRAIL was combined with TPA (data not shown). Therefore, activation of apoptosis through TRAIL receptor 2 (DR5) by Lexatumumab or TRAIL in combination with TPA treatment can promote cell death in TRAIL-resistant LNCaP cells. In contrast, TRAIL receptor 1 (DR4) level is not associated with TPA-enhanced TRAIL-induced apoptosis. Our findings suggest that DR5 mediated pathways are more critical to TPA-enhanced TRAIL-induced apoptosis than the DR4 mediated pathways.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that TPA enhances HGS-ETR2 resistant prostate cancer xenografts by up-regulating c-Fos and repressing c-FLIP(L).
2. Demonstrated that TRAIL receptor DR5 is more functionally important for inducing apoptosis than TRAIL receptor DR4 for TPA enhanced apoptosis.

REPORTABLE OUTCOMES

With support we received from DoD, we have published five manuscripts related to apoptotic pathways and mechanisms of TRAIL-resistance in 2007.

CONCLUSIONS

Apoptotic pathways are altered in initiation and progression of most cancers, including prostate cancer, therefore, targeting apoptotic pathways for treatment of advanced prostate cancer is a rational approach. TRAIL-agonist compounds, like HGS-ETR2, which are effective against cancer cells but spare normal cells are ideal agents to fight cancer, because they have minimal associated cytotoxicity. Currently, HGS-ETR2 is in clinical trials for treatment of various malignancies. Therefore, it is important to differentiate between patients who harbor tumors that are sensitive as opposed to those with resistant tumors to pro-apoptotic agents like HGS-ETR2. In our progress report we have shown that TPA sensitizes resistant prostate cancer cells and xenografts to HGS-ETR2 induced apoptosis.

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Low-Dose 12-*O*-Tetradecanoylphorbol-13-Acetate Enhances Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Induced Apoptosis in Prostate Cancer Cells

Xiaoping Zhang, Wenhua Li, and Aria F. Olumi

Abstract

Purpose: Previously, we have shown that c-Fos/activator protein-1 (AP-1) promotes tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL)–induced apoptosis by repressing the antiapoptotic molecule c-FLIP(L). In this study, we investigated whether synthetic induction of c-Fos/AP-1 by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) converts the phenotype of TRAIL-resistant prostate cancer cells to a TRAIL-sensitive phenotype *in vitro* and *in vivo*.

Experimental Design: Low-dose TPA was used to determine whether LNCaP prostate cancer cells could be converted to a TRAIL-sensitive phenotype in *in vitro* and *in vivo* studies. We also assessed whether TPA enhancement of TRAIL-induced apoptosis varies between androgen-sensitive and androgen-insensitive prostate cancer cells and evaluated the role of TRAIL receptors, DR4 and DR5, in TPA-enhanced TRAIL-induced apoptosis.

Results: We show that the combination of TRAIL with low-dose TPA has no effect on nonmalignant prostate epithelial cells; however, TPA up-regulates most AP-1 proteins and AP-1 activity, reduces c-FLIP(L), and potentiates TRAIL-induced apoptosis. We show that the combination of TPA + TRAIL is effective in promoting apoptosis in both hormone-sensitive LNCaP and hormone-insensitive LNCaP-C4-2 prostate cancer cells. Although TPA enhances the TRAIL-receptor 1 (DR4) level, sensitization of prostate cancer cells seems to be more dependent on TRAIL-receptor 2 (DR5) than TRAIL-receptor 1 levels. *In vivo* xenograft experiments suggest that TPA elevates the expression of c-Fos and reduces c-FLIP(L). Combination of TPA with TRAIL-receptor 2 agonist antibody, lexatumumab, effectively increases apoptosis and reduces LNCaP xenograft tumor burden.

Conclusions: TPA, when combined with the proapoptotic agent TRAIL, is effective in changing the phenotype of some TRAIL-resistant prostate cancer cells to a TRAIL-sensitive phenotype.

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL/Apo2L) has a unique selectivity for triggering apoptosis in many cancer or transformed cells, but with minimal to no effect on most normal cells (1–3); therefore, it is associated with minimal cytotoxicity (4). Recently, TRAIL-like ligands and agonist TRAIL-receptor monoclonal antibodies have entered phase I and II clinical trials with a very limited cytotoxic profile when used systemically in a variety of cancers (5, 6). Therefore, TRAIL-receptor agonists are new proapoptotic pharmaceutical agents with great potential as new cancer therapeutic agents. Although many cancer cells undergo TRAIL-mediated apoptosis, some are resistant to TRAIL. Therefore, we have been investigating mechanisms to overcome TRAIL resistance in cancer cells so that TRAIL-associated compounds can be used effectively in clinical trials.

TRAIL interacts with specific death domain receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), to induce intracellular cytoplasmic formation of the DISC (death-inducing signaling complex; ref. 7). Following the formation of DISC at the intracellular plasma membrane, proapoptotic signals are initiated by caspase-8, which can further activate downstream proapoptotic molecules and subsequent programmed cell death, which may also activate the mitochondrial mediated proapoptotic pathways via cleavage of Bid (8). A key inhibitor of death receptor signaling is c-FLICE–like inhibitory protein (c-FLIP; refs. 9, 10). c-FLIP shows a high level of homology to caspase-8 and caspase-10, but has no protease activity and prevents the formation of a competent DISC by binding to the FADD adaptor protein and competing off caspase-8 (10). We have previously shown that expression of c-FLIP long form (c-FLIP(L)), not c-FLIP short form (c-FLIP(s)), is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis in

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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prostate cancer cells (11). More recently, we have shown that c-Fos, one of the two major components of activator protein-1 (AP-1) family member proteins, has a novel proapoptotic function by priming prostate cancer cells to undergo TRAIL-induced apoptosis (12, 13). We have found that the up-regulation of c-Fos/AP-1 is necessary but insufficient for cancer cells to be sensitive to TRAIL-induced apoptosis. We have also found that c-Fos/AP-1 functions as a proapoptotic molecule by directly repressing the antiapoptotic gene, *c-FLIP(L)*. This finding suggests that strategies to potentiate c-Fos/AP-1 activation and/or inhibit c-FLIP(L) may enhance the efficacy of TRAIL for treatment of various malignancies.

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) has been shown to activate protein kinase C (PKC) and c-jun-NH_2-kinase pathways and the AP-1 proteins (e.g., c-Fos and c-Jun) to promote differentiation, cell cycle arrest, and apoptosis in a variety of cell model systems (14–16). TPA can directly mediate expression of AP-1 genes via serum response element sites at their promoters (16). In addition, TPA can activate PKC and mitogen-activated protein kinases, which will directly or indirectly activate AP-1 proteins and their functions (16). Therefore, TPA can strongly induce the expression of AP-1 proteins. Recently, high-dose TPA alone has been shown to promote apoptosis in androgen-dependent prostate cancer cells (17) and enhance the therapeutic effects of radiation in LNCaP prostate cancer cells (18, 19). More importantly, TPA has been used in a variety of clinical trials to potentiate the effect of chemotherapy (15, 20–22). Therefore, TPA has the potential of enhancing the therapeutic effects of some systemic agents for the treatment of various malignancies.

Because we have found that the activation of c-Fos/AP-1 is necessary for cancer cells to undergo TRAIL-induced apoptosis and TPA is a strong inducer of c-Fos/AP-1, we hypothesized that TPA might sensitize TRAIL-resistant prostate cancer cells to undergo apoptosis after TRAIL treatment. In the present study, we show that TRAIL or a TRAIL-R2 agonist antibody, combined with low-dose TPA, up-regulates AP-1 proteins and its activity, reduces c-FLIP(L) levels, and potentiates apoptosis in TRAIL-resistant LNCaP cells in *in vitro* and *in vivo* experiments. Therefore, TPA, when combined with the proapoptotic agent TRAIL, is effective in changing the phenotype of some TRAIL-resistant prostate cancers to a TRAIL-sensitive phenotype.

Materials and Methods

Materials. Recombinant human TRAIL/TNFSF10 was obtained from R&D System Inc. TRAIL-receptor 1 (DR4) agonist monoclonal antibody, mapatumumab, and TRAIL-receptor 2 (DR5) agonist monoclonal antibody, lexatumumab, were obtained from Human Genome Sciences, Inc. Antibodies to c-Fos, Fos B, Fra-1, Fra-2, Jun B, Jun D, DR4, DR4 RNAi, and horseradish peroxidase–conjugated secondary antibodies (goat–anti-mouse, goat–anti-rabbit, and goat–anti-rat antibodies) were obtained from Santa Cruz Biotechnology, Inc. Antibody to c-Jun was from Cell Signaling. Antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Abcam. Antibodies to c-FLIP and DR5 were from Apotech Corp. TPA was from Sigma.

Cell culture material, cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, apoptosis by Annexin V-FITC, Western blot assays, transfection of plasmids, and RNAi and luciferase assay. References for material and techniques can be found from our previously published works (5, 12, 13). DR4, DR5, and their vector pcDNA3.1 were from Dr. Roya Khosravi-Far's laboratory (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA).

Establishment of mouse s.c. xenograft and experimental design. LNCaP cells 1×10^6 were mixed with Matrigel (BD Biosciences) according to manufacturer's protocol and injected into the posterior trunk of each athymic nude mice (Charles River Laboratories). Nine weeks after injection, 28 mice with visible tumor mass were randomly divided into four groups (each has seven mice): vehicle group, lexatumumab (Lexa) group, TPA group, and TPA + Lexa group, and treated with saline, TPA (100 ng/g), Lexa (10 μg/g), and TPA (100 ng/g) + Lexa (10 μg/g) via tail vein twice a week, respectively. Mouse body weight and tumor area were measured twice a week. Four weeks after treatment, all animals were euthanized, and xenografts were harvested and assessed for tumor weight, apoptosis [terminal nucleotidyl transferase–mediated nick end labeling (TUNEL)], and Western blot for c-Fos and c-FLIP. Tissue samples for Western blot analysis were preserved in liquid nitrogen and then prepared in radioimmunoprecipitation assay buffer with 2% SDS. All experiments were approved by the Institutional Animal Care and Use Committee at our institution.

TUNEL labeling to assess the apoptotic cells was done using a commercial kit according to the manufacturer's instruction (Promega Co.). Background reactivity was determined by processing slides in the absence of terminal deoxynucleotidyl transferase (negative control); maximum reactivity was observed by preincubating the tissue sections with DNase I to confirm the quality of the specimen and availability of protocol. Tissue sections were mounted with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.) to counterstain the nuclei. At least three microscopic views (each with at least 200 cells) from each sample were captured with a Nikon TE300 microscope and analyzed by counting positive rate. Positive apoptotic rate is defined as the ratio of green staining within the nuclear area to the total nuclear staining (blue).

Statistical analysis. GraphPad Instat software (version 3.0) was used for all statistical analyses. For apoptosis, cell viability, tumor area, and tumor volume assessments, Kruskal-Wallis test (nonparametric ANOVA) was used to compare each treatment group.

Results

Low-dose TPA sensitizes LNCaP cells to TRAIL-induced apoptosis. Although PC3 prostate cancer cells are sensitive to TRAIL-induced apoptosis, LNCaP cells and nontumorigenic and immortalized BPH-1 cells (benign prostatic hyperplasia cells) are resistant to the proapoptotic effects of TRAIL (Fig. 1A). Because high-dose TPA can induce apoptosis in androgen-dependent prostate cancer cells (17, 23), we focused on identifying a low dose of TPA, which does not directly induce apoptosis as a single agent. We found that TPA at 100 ng/mL did not induce cell death in LNCaP or BPH-1 cells (Fig. 1B). However, at higher concentrations (1,000 to 10,000 ng/mL), TPA induced cell death in LNCaP prostate cancer cells, but not the nontumorigenic transformed BPH-1 cells (Fig. 1B).

Next, we examined whether a nontoxic dose of TPA (100 ng/mL) can enhance the efficacy of TRAIL-induced apoptosis. We found that TPA combined with TRAIL significantly increased apoptosis in the TRAIL-resistant LNCaP cells as shown by Annexin V (Fig. 1C) and cell viability (Fig. 1D) assays. However, TPA, combined with TRAIL, did not significantly affect the cell viability of BPH-1 cells (Fig. 1D). Therefore, a non-proapoptotic dose of TPA can enhance TRAIL-induced apoptosis in LNCaP cells, but not in nonmalignant transformed prostate cells like BPH-1.

c-Fos/AP-1 is required for TPA enhancement of TRAIL-induced apoptosis in LNCaP cells. To assess whether low-dose TPA can

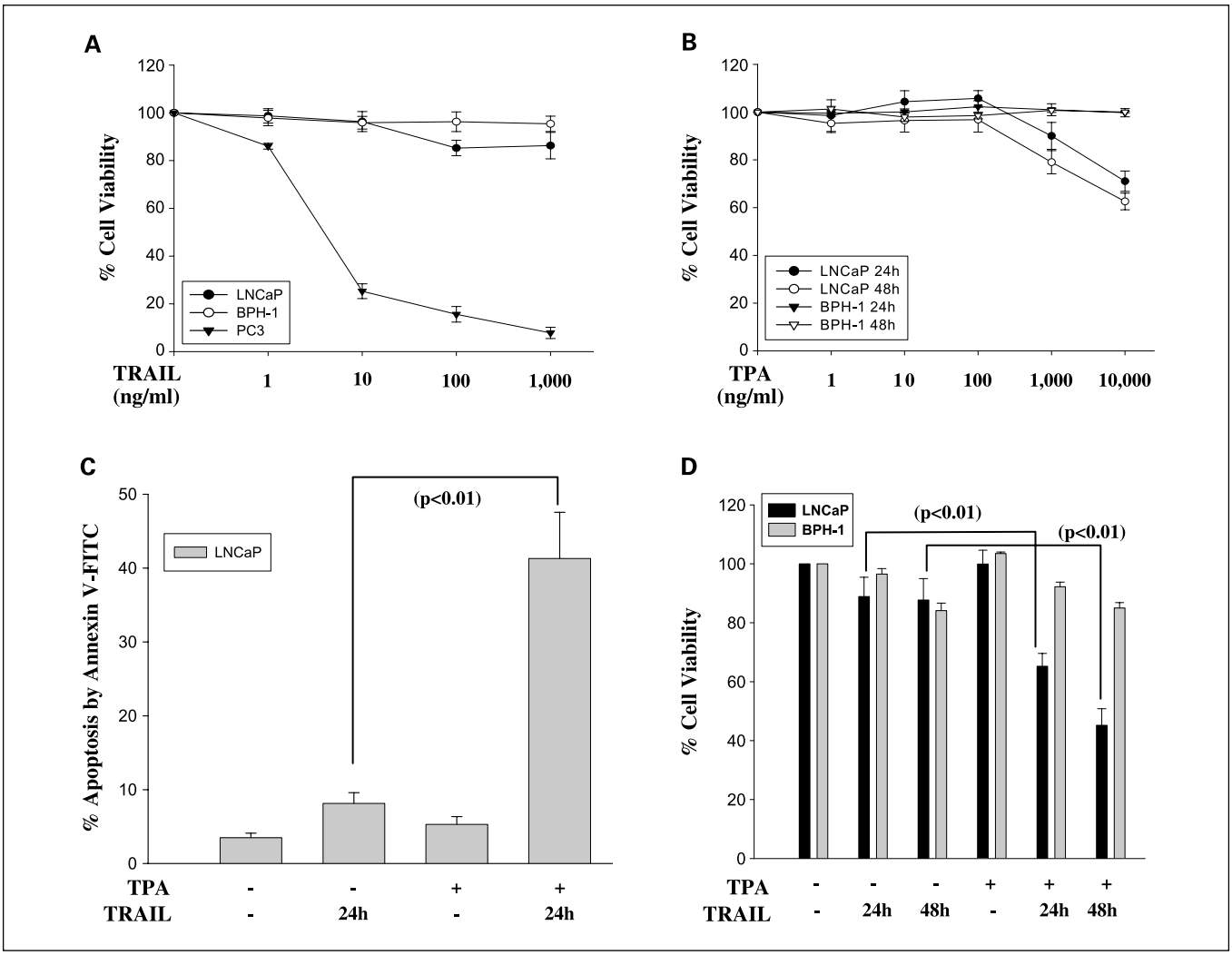

Fig. 1. Low-dose TPA enhances TRAIL-induced apoptosis in LNCaP cells. *A,* cell viability of PC3, LNCaP, and BPH-1 cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were treated with TRAIL for 24 h. *B,* cell viability of LNCaP and BPH-1 cells treated with different doses of TPA for 24 h and 48 h. *C,* percentage of apoptotic cells was measured by Annexin V – FITC staining. *D,* cell viability of LNCaP and BPH-1 cells. Cells in (*C*) and (*D*) were treated with TPA alone (100 ng/mL) or pretreated with TPA (100 ng/mL) for 24 h and then treated with TRAIL (100 ng/mL) for the indicated times. Bars, SD from at least three independent experiments.

induce the expression of AP-1 family members and AP-1 activity, we examined for protein expression and AP-1 activity in LNCaP cells. We found that TPA alone or TPA combined with TRAIL increased not only the expression of c-Fos, one of the two major members of AP-1 family, but also other AP-1 family members like Fos B, Fra-1, Fra-2, Jun B, and Jun D (Fig. 2A). However, c-Jun protein levels, another major AP-1 family member protein, were not changed significantly. In accordance with our immunoblot findings (Fig. 2A), TPA alone or TPA combined with TRAIL increased AP-1 activity (Fig. 2B). Therefore, these results suggest that TPA increases the level of some AP-1 proteins and enhances AP-1 – related gene activity.

Because c-Fos and c-Jun are two major components of the AP-1 complex (24) and the expression of c-Jun was not changed before and after TPA treatment (Fig. 2A), we determined whether the induction of c-Fos/AP-1 by TPA was required for TRAIL-induced apoptosis in resistant prostate cancer cells. We used a dominant negative form of c-Fos/AP-1, A-Fos (25), to inhibit c-Fos/AP-1. We found that A-Fos inhibited TPA-induced AP-1 activity (Fig. 2C, *top, two rightmost columns*). In conjunction with reduced AP-1 activity, TPA did not enhance the ability of TRAIL to promote apoptosis when the activity of c-Fos/AP-1 was inhibited by the dominant negative A-Fos (Fig. 2C, *bottom, rightmost column*). Therefore, the activity of c-Fos/AP-1 is necessary for TPA to enhance TRAIL-induced apoptosis.

In our previous studies, we have shown that c-Fos/AP-1 was necessary for cancer cells to be sensitive to TRAIL-induced apoptosis (12, 13). Furthermore, we have shown that one of the major mechanisms for c-Fos/AP-1 to promote apoptosis is through cytoplasmic to nuclear translocation and direct inhibition of c-FLIP(L) gene via binding to its promoter region (12, 13). Similarly, in the current study, we also found that the combination of TPA and TRAIL reduced both mRNA of c-FLIP(L) and protein levels (Fig. 2D), which confirms that the regulation of c-FLIP(L), at least partially, occurs at the transcriptional level. It is noteworthy that although TPA alone increased AP-1 activity by 3-fold (Fig. 2B), it did not reduce c-FLIP(L) levels (Fig. 2D). However, TPA combined with TRAIL

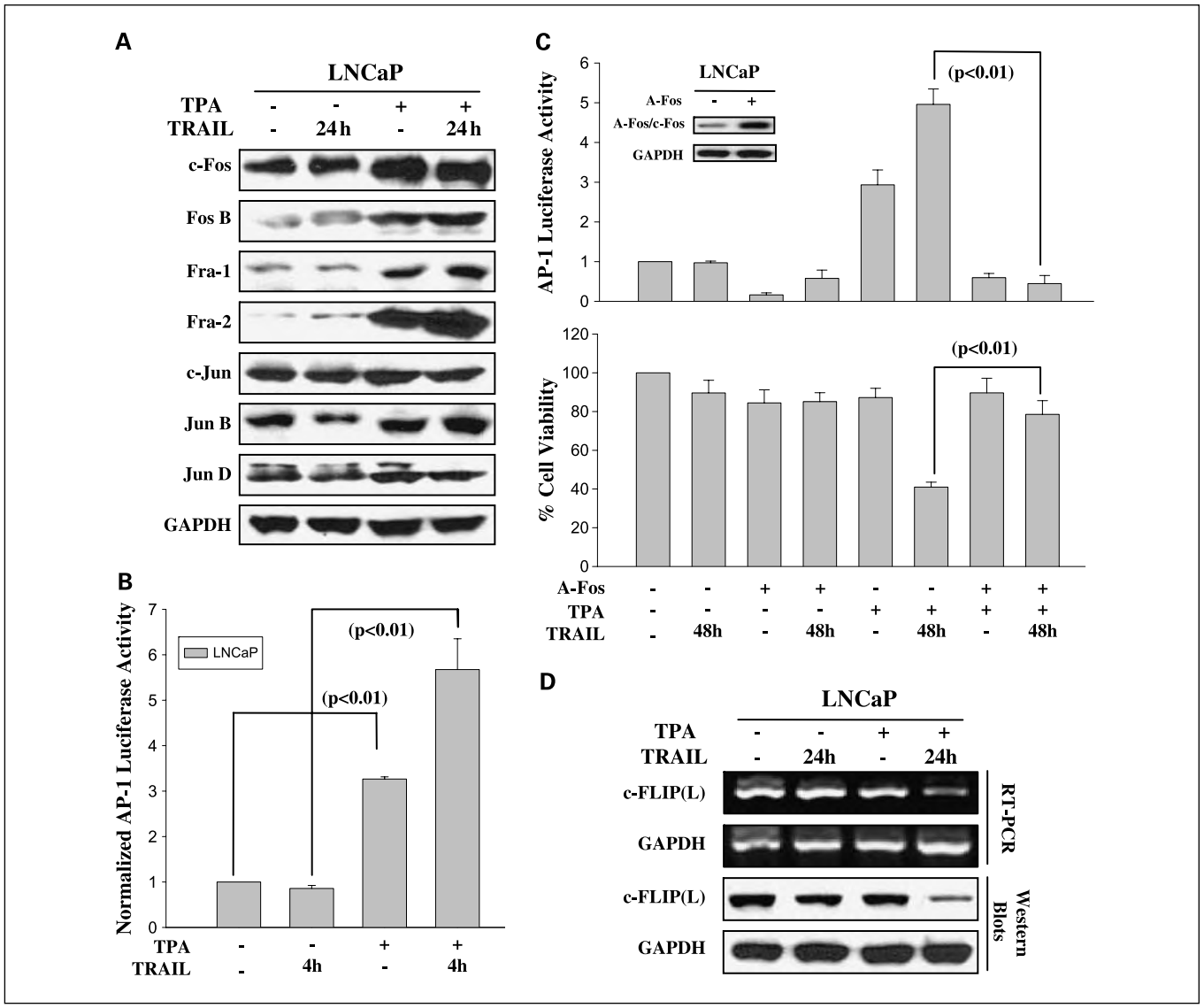

Fig. 2. TPA-induced AP-1 activity is necessary for TRAIL-induced apoptosis in resistant prostate cancer cells. *A,* TPA increases the levels of several AP-1 family-related proteins. *B,* AP-1 luciferase activity is increased in LNCaP cells treated with TPA. *C,* top, AP-1 luciferase activity; bottom, cell viability assays. Top inset, Western blot represents expression of A-Fos and c-Fos after mock or A-Fos transfections. Note that the antibody recognizes both A-Fos (dominant negative) and endogenous c-Fos. Cells were transfected with A-Fos for 24 h before TPA and TRAIL treatments. *D,* reverse transcription-PCR and Western blots for c-FLIP(L). LNCaP cells treated with TPA alone (100 ng/mL) or pretreated with TPA (100 ng/mL) for 24 h then treated with TRAIL (100 ng/mL) for the indicated time. GAPDH represents loading control for mRNA or proteins. Bars, SD from at least three independent experiments.

increased AP-1 activity more and reduced the expression of c-FLIP(L). Therefore, the activation of c-Fos/AP-1 is necessary to sensitize TRAIL-resistant prostate cancer cells but insufficient to promote apoptosis. We believe that the repression of *c-FLIP(L)* by c-Fos is one of the molecular mechanisms that promotes TRAIL-induced apoptosis (11–13).

TPA enhances lexatumumab-induced apoptosis in LNCaP cells in vivo. To determine whether TPA can enhance TRAIL-induced apoptosis *in vivo*, we used a TRAIL receptor agonist monoclonal antibody, lexatumumab, that is currently in clinical trials for treatment of various malignancies (5, 6). Lexatumumab induces apoptosis via similar pathways as recombinant TRAIL (26, 27). We found that lexatumumab had equivalent sensitivity toward TRAIL-resistant (LNCaP) and TRAIL-sensitive (PC3) cells as recombinant TRAIL (Figs. 1A and 3A). Therefore, using lexatumumab as opposed to recombinant TRAIL for our *in vivo* experiments is more clinically relevant at the current time (28).

Nine weeks after LNCaP xenograft implantation, 28 mice with equivalent tumor sizes (Fig. 3B, week 0) were randomly divided into four treatment groups (7 mice per group): vehicle (saline), lexatumumab (Lexa, 10 μg/g), TPA (100 ng/g) or TPA + Lexa groups. Each treatment group was treated twice per week for 4 weeks via tail-vein injection with the indicated regimen. Before initiating therapy, the average tumor area for each group was 73.3 ± 12.0, 82.6 ± 12.8, 73.1 ± 19.2, and 80.4 ± 5.5 mm^2 for the vehicle, Lexa, TPA, and TPA + Lexa groups, respectively (Fig. 3B; *P* value, not significant). However, on weeks 3 and 4 after initiating therapy, the group treated with TPA + Lexa had a much lower tumor area than any of

the other treatment groups (mean tumor area for week 3: 50.6 ± 10.6 mm^2; $P < 0.05$; for week 4: 41.7 ± 11.2 mm^2, $P = 0.01$; Fig. 3B). The average tumor weight of the animals treated with vehicle, Lexa, or TPA was comparable, whereas the median tumor weight of the animals treated with TPA + Lexa was 5-fold lower as compared with the other groups [Tumor weights (median ± SE): vehicle 856 ± 415 mg; Lexa 1,306 ± 257 mg; TPA 1,297 ± 305 mg; TPA+ Lexa 150 ± 65 mg; Fig. 3C, $P < 0.05$].

To examine whether reduced tumor volume and weight in TPA + Lexa–treated animals is associated with increased apoptosis rate, the xenografts were analyzed for TUNEL staining. We found that the TPA + Lexa–treated group had a significantly higher rate of TUNEL staining compared with other treatment groups (Fig. 4A and Supplementary Fig. S1).

Because our *in vitro* studies have shown that TPA promotes AP-1–related proteins, we evaluated the expression of c-Fos in the xenografts from different treatment groups. Similar to our *in vitro* findings, we found that c-Fos protein levels were elevated in xenografts that were exposed to TPA or TPA + Lexa (Fig. 4B). In our previous studies, we have shown that c-Fos binds and represses the transcription of the antiapoptotic protein, c-FLIP(L) (11–13). Therefore, we wished to evaluate protein levels of c-FLIP(L) in the xenografts. We found that c-FLIP(L) protein levels were reduced in the animals that were treated with either TPA or TPA + Lexa (Fig. 4B). Although c-FLIP(L) levels were reduced in the TPA-treated group, the xenograft volume and weight were not significantly affected in the TPA-treated group, a finding that is different from our *in vitro* studies (Fig. 2D), suggesting that reduction of c-FLIP(L) alone was not sufficient to affect tumor burden *in vivo*. To investigate whether the differences in c-FLIP(L) expression *in vitro* and *in vivo* are not secondary to using TRAIL (for the *in vitro* studies) and the TRAIL receptor agonist, lexatumumab (for the *in vivo* studies), we used lexatumumab in combination with TPA in *in vitro* studies. We found that both lexatumumab and TRAIL, when used alone or in combination with TPA, had similar effects on AP-1 activity and c-FLIP(L) levels (Figs. 2B and D and 4C). Therefore, these results underscore the differences between *in vitro* and *in vivo* studies (29).

Next, we evaluated the possible toxicity of our treatments in the animals. Because lexatumumab is a humanized monoclonal antibody, we did not expect that it would activate the mouse TRAIL receptor and lead to side effects in the mouse. However, to examine the possible side effects of TPA, animals were weighed twice per week during the study period. We did not observe any difference between the total body weight of the animals in either the vehicle-treated or any of the other treatment groups. In addition, at the completion of the study when the mice were euthanized, liver, lung, kidney, heart, and brains of the mice were harvested and assessed by H&E microscopic evaluation. We did not observe any evidence of toxicity or necrotic changes in any of the organs examined (Supplementary Fig. S1). Therefore, in TRAIL-resistant prostate cancer cell, LNCaP, the combination of low-dose TPA with the TRAIL receptor 2-agonist antibody, lexatumumab, can dramatically decrease tumor area and weight *in vivo* and significantly increase apoptosis by increasing c-Fos levels and reducing c-FLIP(L) levels.

TPA enhances TRAIL-induced apoptosis in androgen-independent prostate cancer cells. Because a majority of the morbidity and mortality associated with prostate cancer is secondary to the progression from an androgen-dependent to an androgen-independent state and inefficacy of currently available systemic regimens (30), we examined whether the combination of TPA and TRAIL is effective against TRAIL-resistant androgen-independent prostate cancer cells. High-dose TPA has been shown to induce apoptosis in the androgen-dependent LNCaP

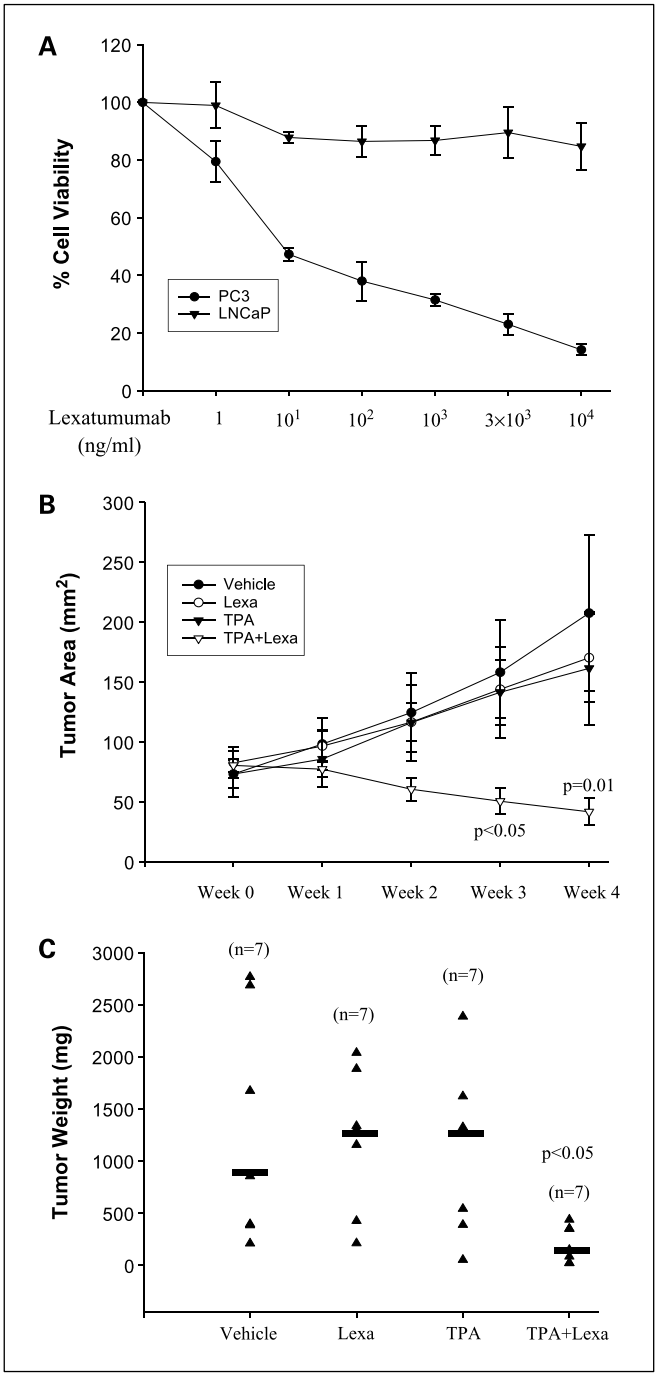

Fig. 3. Combination of TPA and lexatumumab reduces tumor burden in LNCaP xenografts. *A,* cell viability of PC3 and LNCaP cells after treatment with lexatumumab (Lexa). Bars, SD. *B,* average tumor area during the 4-week treatment in vehicle, Lexa, TPA and TPA + Lexa groups. Week 0, tumor sizes before initiating therapy. *C,* scatter plot of tumor weight. Triangle, tumor weight of a xenograft; black bars, median weight for the tumors in each group ($n = 7$ per group). Bars, SD. *P* values represent comparison of the TPA + Lexa group to the other treatment arms.

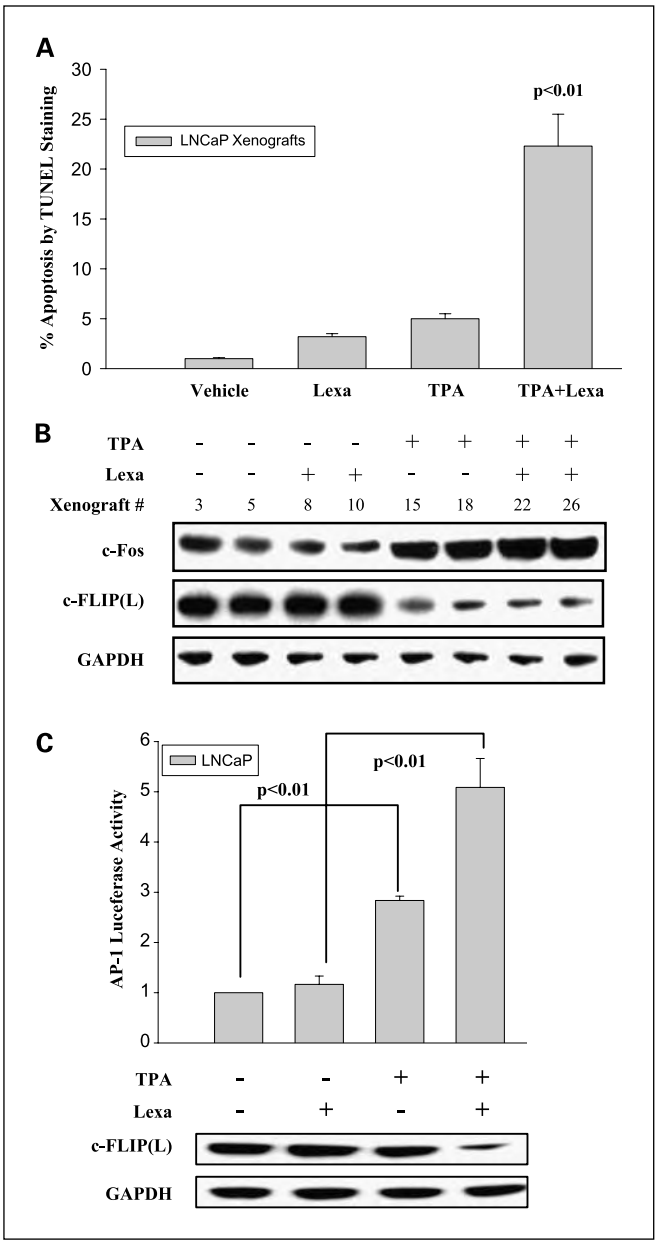

Fig. 4. Combination of TPA and lexatumumab increases apoptosis in LNCaP xenografts. *A,* TUNEL assay; percentage of apoptotic cells was measured by evaluating three randomly selected microscopic fields at 40× magnification. Bars, SD. *P* value is measured by comparisons between TPA + Lexa group and other treatment groups. *B,* Western blot analysis of c-Fos and c-FLIP(L) in LNCaP tumor xenografts. *C, in vitro* AP-1 activity and c-FLIP(L) protein levels for LNCaP cells. Cells were pretreated with TPA (100 ng/mL) for 24 h and then treated with lexatumumab (10 μg/mL) for 4 h when assessing AP-1 activity levels and for 24 h when assessing for c-FLIP(L) protein levels.

prostate cancer cells, whereas TPA is thought to be ineffective in androgen-independent prostate cancer cells (17, 18). Because high-dose TPA is associated with tumor progression properties (15, 21), we determined whether low-dose TPA, when combined with TRAIL, can also sensitize androgen-independent prostate cancer cells that are resistant to TRAIL-induced apoptosis. We found that C4-2 cells, an androgen-independent subline of LNCaP cells (31), were resistant to TRAIL-induced apoptosis, a finding similar to the parental LNCaP cells, which are androgen dependent (Figs. 1A and 5A). Next, we determined whether TPA can enhance the proapoptotic properties of TRAIL. We found that the combination of TPA with TRAIL converted the phenotype of TRAIL-resistant C4-2 cells to a TRAIL-sensitive phenotype as evidenced by increased apoptotic rate (Fig. 5B) and reduced cell viability (Fig. 5C). In concert with our earlier findings in the androgen-dependent LNCaP cells (Fig. 2B), the enhancement of TRAIL-induced apoptosis was associated with increased AP-1 activity in the androgen-independent C4-2 cells (Fig. 5D). Therefore, low-dose TPA is capable of converting the phenotype of TRAIL-resistant prostate cancer cells in both androgen-dependent and androgen-independent states by increasing AP-1 gene activity.

TPA enhancement of TRAIL-induced apoptosis is independent of DR4 levels. Treatment of LNCaP cells with TRAIL does not alter the protein levels of the DR4 or DR5 TRAIL receptors (Fig. 6A, *top*). Some investigators have suggested that TPA enhances the expression of TRAIL receptor DR4 via an AP-1–dependent mechanism (32). However, it is unclear whether increased levels of DR4 are associated with the enhancement of TRAIL-induced apoptosis. Similar to others (32), we found that TPA increased TRAIL receptor DR4 levels, but not DR5 levels (Fig. 6A, *bottom*). Next, we determined whether DR4 and/or DR5 play a functional role in TPA-enhanced TRAIL-induced apoptosis. Because recombinant TRAIL activates both DR4 (TRAIL-R1) and DR5 (TRAIL-R2) by promoting the trimerization of these cell surface receptors, we used fully human monoclonal agonist antibodies specifically targeted against TRAIL-R1 (mapatumumab) and TRAIL-R2 (lexatumumab; refs. 5, 6). We found that mapatumumab alone or in combination with TPA did not enhance TRAIL-induced cell death in LNCaP cells (Fig. 6B). In contrast, lexatumumab, when combined with TPA, promoted cell death and reduced cell viability in the TRAIL-resistant prostate cancer cells (Fig. 6B), which is similar to the results using soluble TRAIL combined with TPA (Fig. 1D) and compatible with our xenograft *in vivo* studies (Fig. 3B and C). Furthermore, we examined whether the inhibition of DR4 by RNAi (Fig. 6C, *inset*) or ectopic expression of DR4 (Fig. 6D, *inset*) would alter sensitivity to TRAIL-induced apoptosis. We found that neither inhibition of DR4 (Fig. 6C) nor increased DR4 levels (Fig. 6D) were associated with TRAIL-induced or TPA-enhanced TRAIL-induced cell death (Fig. 6C and D). Conversely, overexpression of DR5 alone sensitized LNCaP cells to TRAIL-induced apoptosis, which was even further enhanced when TRAIL was combined with TPA (Supplementary Fig. S2). Therefore, the activation of apoptosis through TRAIL receptor 2 (DR5) by lexatumumab or TRAIL, in combination with TPA treatment, can promote cell death in TRAIL-resistant LNCaP cells. In contrast, TRAIL receptor 1 (DR4) level is not associated with TPA-enhanced TRAIL-induced apoptosis. Our findings suggest that DR5-mediated pathways are more critical to TPA-enhanced TRAIL-induced apoptosis than the DR4-mediated pathways.

Discussion

Prostate cancer is the second leading cause of death in men, accounting for 232,900 new cases annually (33). Typically, localized prostate cancer is treated effectively with surgery or radiotherapy and for carefully selected cases with watchful waiting (34). However, advanced hormone refractory prostate

cancer is fatal and accounts for 30,350 deaths annually (33). The proapoptotic agent, TRAIL, has great potential as an antitumor agent because it selectively induces apoptosis in cancer cells (1–3). Although many cancer cells are sensitive to TRAIL-induced apoptosis, some develop resistance. Many groups have been investigating the synergistic effects of different drugs in combination with TRAIL to overcome the resistance developed by cancer cells (35–42). Previously, we have shown that the activation of c-Fos/AP-1 is a necessary component for cancer cells to undergo TRAIL-induced apoptosis (12, 13). Therefore, in the present study, we investigated whether the activation of c-Fos/AP-1 by a synthetic compound, TPA, may convert the TRAIL-resistant prostate cancer cells to become TRAIL sensitive. We show that TRAIL combined with low-dose TPA effectively sensitizes TRAIL-resistant prostate cancer cells to undergo apoptosis *in vitro* and *in vivo*. Low-dose TPA sensitizes TRAIL-resistant prostate cancer cells by up-regulating the AP-1 family proteins and AP-1 activity. Moreover, the combination of TRAIL with low-dose TPA enhances cell death in androgen-dependent and androgen-independent prostate cancers, but not in nonmalignant transformed BPH-1 cells.

From our current and past experience (11), TRAIL sensitivity does not seem to be related to androgen dependency of prostate cancer cells. For example, we have found that some androgen-independent cells such as PC3 and DU145, cells are sensitive to TRAIL, whereas other androgen-independent cells such as C4-2 are resistant to TRAIL (ref. 11; Fig. 5). Conversely, the androgen-dependent LNCaP and CWR22 cells are resistant to TRAIL (Fig. 1 and data not shown). Therefore, TPA can enhance TRAIL-induced apoptosis in resistant prostate cancer cells regardless of their androgen dependency state. Clinically, this is an important distinction because low-dose TPA can potentially enhance the proapoptotic activity of TRAIL in prostate cancer patients in androgen-dependent and androgen-independent states, therefore making this combination therapy more widely available for prostate cancer patients with advanced disease.

TRAIL binds at least five cell surface receptors: DR4, DR5, and three decoy receptors DcR1, DcR2, and osterprotegerin (43). Only binding to DR4 or DR5 initiates TRAIL-induced apoptosis. When TRAIL and TRAIL receptors were initially identified, it was logical to suspect that expression levels of TRAIL receptors may contribute greatly to TRAIL sensitivity

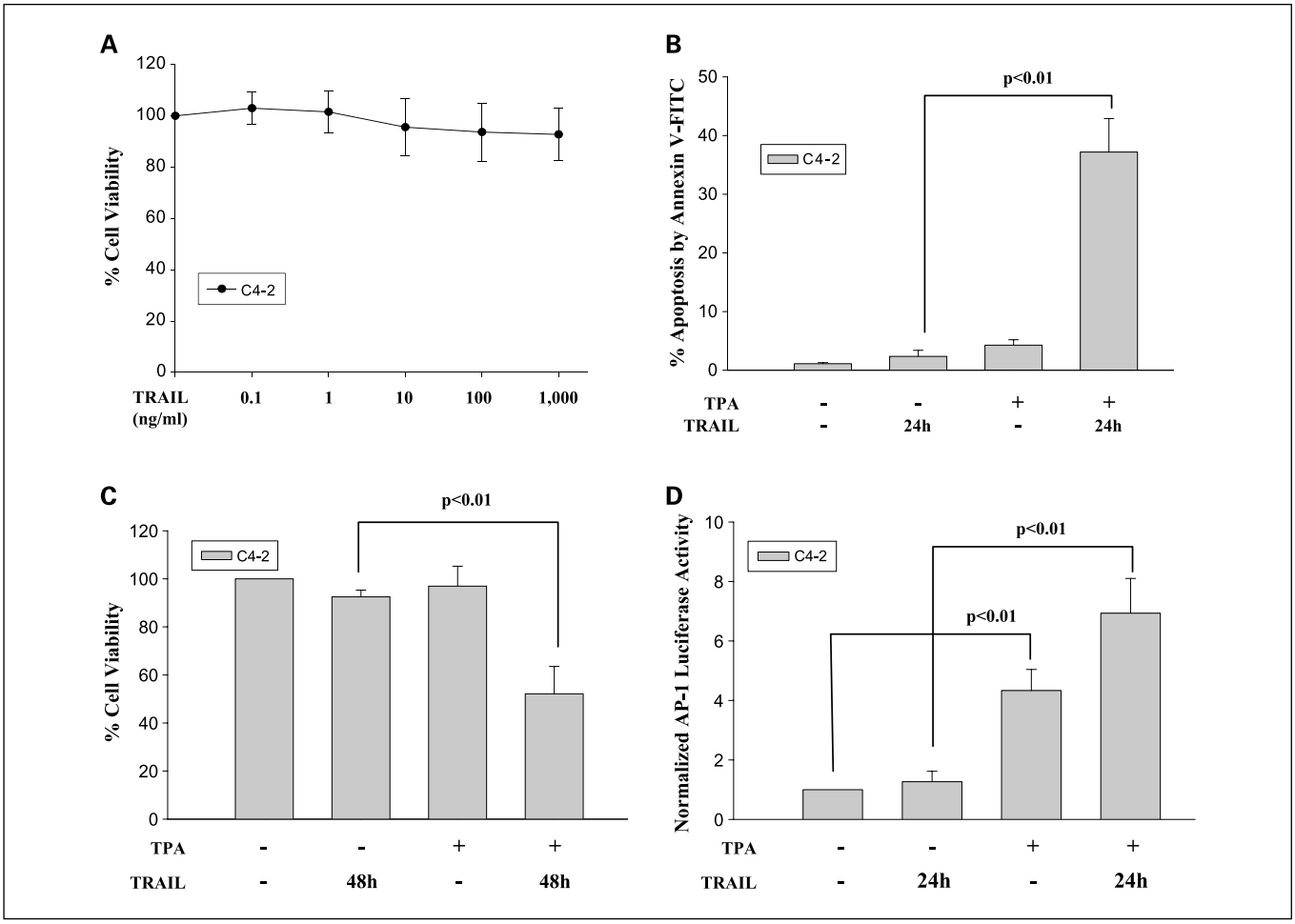

Fig. 5. TPA enhances TRAIL-induced apoptosis in androgen-independent prostate cancer C4-2 cells. *A,* cell viability of C4-2 cells treated with increasing doses of TRAIL for 48 h. Percentage of apoptotic cells measured by (*B*) Annexin V – FITC staining, (*C*) cell viability, and (*D*) AP-1 luciferase activity of C4-2 cells treated with TPA and/or TRAIL. C4-2 cells in (*B*) to (*D*) were treated with TPA alone (100 ng/mL) or pretreated with TPA (100 ng/mL) for 24 h followed by TRAIL (100 ng/mL) treatment for the indicated times. Bars, SD from at least three independent experiments.

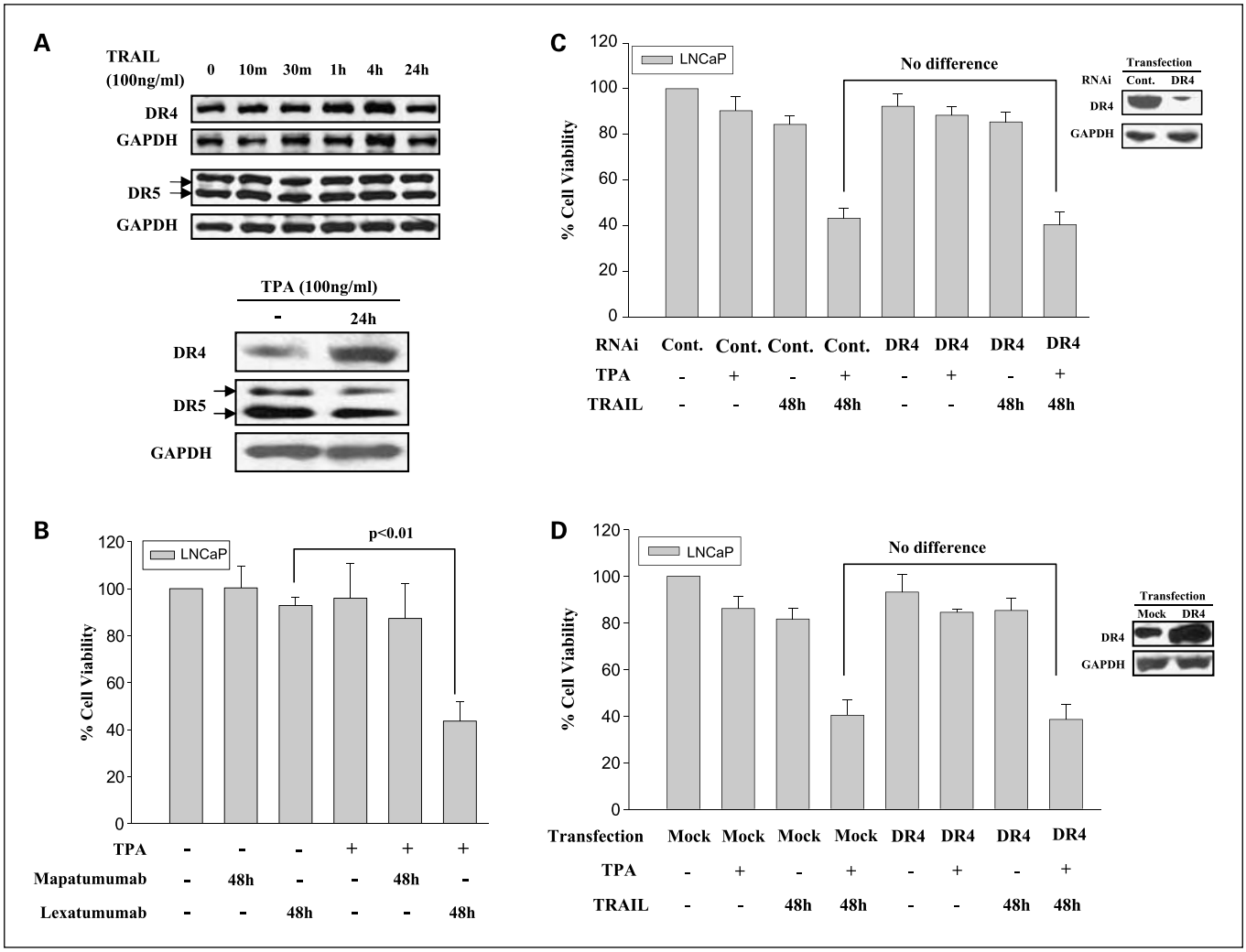

Fig. 6. TPA enhancement of TRAIL-induced apoptosis is independent of DR4 levels. *A,* Western blots for DR4 and DR5 levels in LNCaP cells after treatment with TRAIL or TPA. *B,* cell viability of LNCaP cells treated with TPA alone (100 ng/mL) or pretreated with TPA (100 ng/mL) for 24 h followed by treatment with the DR4 agonist, mapatumumab (10 μg/mL), or the DR5 agonist, lexatumumab (10 μg/mL), for another 48 h. *C,* cell viability for LNCaP cells after transfecting DR4 RNAi for 16 h and then pretreated with TPA for 24 h, followed by treatment with TRAIL for 48 h. *D,* cell viability of LNCaP cells, which were determined after ectopic expression of DR4 for 24 h, followed by pretreatment with TPA for 24 h followed by treatment with TRAIL for an additional 48 h. *C* and *D,* insets, Western blots for DR4. GAPDH is used as loading control. Bars, SD from at least three independent experiments.

(44). However, later studies have shown that there are no significant associations between TRAIL sensitivity and expression level of TRAIL receptors (45). Recently, it has been shown that TPA enhances the expression of DR4 by increasing AP-1 binding at the DR4 promoter region (32); however, it has been unclear whether increased DR4 levels by TPA can potentiate the sensitivity of prostate cancer cells to TRAIL-induced apoptosis. In this study, we showed that TRAIL treatment alone does not change DR4 and DR5 levels (Fig. 6A), and we also confirmed that TPA increased DR4 protein levels, as has been shown by others (32). However, we found that increased DR4 levels alone are not associated with enhancing TRAIL-induced apoptosis because ectopic expression of DR4 in LNCaP cells did not potentiate TRAIL-induced apoptosis or TPA-enhanced TRAIL-induced apoptosis (Fig. 6D). To investigate whether DR4 or DR5 may be more important for TRAIL-induced apoptosis when combined with TPA, we used human monoclonal agonist antibodies, mapatumumab and lexatumumab, which target TRAIL-R1 and TRAIL-R2, respectively. We found that lexatumumab (TRAIL-R2 agonist) and not mapatumumab (TRAIL-R1 agonist) is capable of promoting cell death when combined with low-dose TPA. In addition, ectopic expression of DR5 sensitizes LNCaP cells to TRAIL-induced apoptosis particularly when combined with TPA (Supplementary Fig. S2). Therefore, TRAIL-R2 cell surface receptor pathway is preferentially activated in this prostate model system during TPA-enhanced TRAIL-induced apoptosis. Our findings are further supported by a recent report that DR5 has a greater contribution to TRAIL-induced apoptosis than DR4 (46).

Many approaches have been employed to overcome TRAIL resistance in cancer cells, notably by combination therapy of TRAIL with chemotherapy or radiotherapy (40–42). A concern about using TPA is its potential toxicity and tumor-promoting properties. Some studies have shown that TPA can act as a tumor promoter in skin tumorigenesis with relatively high

concentrations (2.5 nmol/L; refs. 47, 48). In other studies, TPA has been shown to promote apoptosis as a single agent in androgen-dependent prostate cancer cells at concentrations of 10 to 50 nmol/L (17, 18, 23), concentrations that are 60- to 300-fold higher than the one used in our *in vitro* and *in vivo* studies (i.e., 100 ng/mL for *in vitro* studies or 100 ng/g for *in vivo* studies is equal to 0.162 nmol/L). In addition, TPA has been used successfully in patients with refractory hematologic malignancies in phase I clinical trails in China and the United States (15, 21, 22). In our study, we specifically focused on using a low dose of TPA to minimize its potential toxicity. Assessing the animals in our *in vivo* studies by body weight and histology of multiple different organs did not show any grossly detectable toxic effects. In addition, recent clinical trials that have used lexatumumab for treatment of various malignancies as a single agent have not been associated with any significant toxicity (5, 6, 28).

In conclusion, we show that TPA activates AP-1 family of proteins and enhances TRAIL-induced apoptosis in both androgen-dependent and androgen-independent prostate cancer cells. In our system, the activation of the DR5 TRAIL receptor may play a more important role than activation of the DR4 TRAIL receptor. Further studies are required to determine whether the combination of TPA with TRAIL agonist compounds is suitable for patients with advanced prostate cancer.

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